Epigenetic modulation of tenascin C in the heart: implications on myocardial ischemia, hypertrophy and metabolism

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Abstract: BACKGROUND Tenascin C (TN-C) is considered to play a pathophysiological role in maladaptive left ventricular remodeling. Yet, the mechanism underlying TN-C-dependent cardiac dysfunction remains elusive. METHOD The present study was designed to investigate the effect of hypoxia and hypertrophic stimuli on TN-C expression in H9c2 cells and its putative regulation by epigenetic mechanisms, namely DNA promoter methylation and microRNAs. In addition, rats subjected to myocardial infarction (MI) were investigated. H9c2 cells were subjected to oxygen and glucose deprivation; incubated with angiotensin II (Ang II); or human TN-C (hTN-C) purified protein. Hypertrophic and fibrotic markers, TN-C promoter methylation as well as mir-335 expression were assessed by reverse transcription and quantitative polymerase chain reaction while TN-C protein levels were assessed by ELISA. RESULTS Tn-C mRNA expression was markedly increased by both oxygen and glucose deprivation and Ang II ($P < 0.01$, respectively). In addition, Ang-II-dependent TN-C upregulation was explained by reduced promoter methylation ($P < 0.05$). Cells treated with hTN-C displayed upregulation of Bnp, Mmp2, -Mhc, integrin 6 and integrin 1. Furthermore, hTN-C treated cells showed a significant reduction in adenosine monophosphate and adenosine triphosphate levels. In vivo, plasma and myocardial TN-C levels were increased 7 days post MI ($P < 0.05$, respectively). This increment in TN-C was accompanied by upregulation of mir-335 ($P < 0.01$). In conclusion, both hypoxic and hypertrophic stimuli lead to epigenetically driven TN-C upregulation and subsequent impairment of cellular energy metabolism in cardiomyoblasts. CONCLUSION These findings might enlighten our understanding on maladaptive left ventricular remodeling and direct towards a strong involvement of TN-C.

DOI: https://doi.org/10.1097/HJH.0000000000002097

Original Article

Epigenetic modulation of tenascin C in the heart: implications on myocardial ischemia, hypertrophy and metabolism

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Background: Tenascin C (TN-C) is considered to play a pathophysiological role in maladaptive left ventricular remodeling. Yet, the mechanism underlying TN-C-dependent cardiac dysfunction remains elusive.

Method: The present study was designed to investigate the effect of hypoxia and hypertrophic stimuli on TN-C expression in H9c2 cells and its putative regulation by epigenetic mechanisms, namely DNA promoter methylation and microRNAs. In addition, rats subjected to myocardial infarction (MI) were investigated. H9c2 cells were subjected to oxygen and glucose deprivation; incubated with angiotensin II (Ang II); or human TN-C (hTN-C) purified protein. Hypertrophic and fibrotic markers, TN-C promoter methylation as well as mir-335 expression were assessed by reverse transcription and quantitative polymerase chain reaction while TN-C protein levels were assessed by ELISA.

Results: Tn-C mRNA expression was markedly increased by both oxygen and glucose deprivation and Ang II ($P < 0.01$, respectively). In addition, Ang-II-dependent TN-C upregulation was explained by reduced promoter methylation ($P < 0.05$). Cells treated with hTN-C displayed upregulation of Bnp, Mmp2, β-Mhc, integrin α6 and integrin β1. Furthermore, hTN-C treated cells showed a significant reduction in adenosine monophosphate and adenosine triphosphate levels. *In vivo*, plasma and myocardial TN-C levels were increased 7 days post MI ($P < 0.05$, respectively). This increment in TN-C was accompanied by upregulation of mir-335 ($P < 0.01$). In conclusion, both hypoxic and hypertrophic stimuli lead to epigenetically driven TN-C upregulation and subsequent impairment of cellular energy metabolism in cardiomyoblasts.

Conclusion: These findings might enlighten our understanding on maladaptive left ventricular remodeling and direct towards a strong involvement of TN-C.

Keywords: epigenetics, hypertrophy, hypoxia, miRNAs, matrix metalloproteinase, tenascin C

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; Ang II, angiotensin II; CMC, cardiomyocyte; ECM, extracellular matrix; hTN-C, human TN-C protein; LCA, left coronary artery; MI, myocardial infarction; MMP, matrix metalloproteinase; OGD, oxygen glucose deprivation; PCr, phosphocreatine; TN-C, tenascin C

INTRODUCTION

Left ventricular remodeling describes changes in form, shape and function following chronic pressure or volume overload, ultimately ensuing to left ventricular dilation and heart failure. These changes occur at the level of the individual cardiomyocyte (CMC) and the extracellular matrix (ECM) components, thereby leading to re-expression of fetal genes, alterations in expression of proteins involved in excitation–contraction coupling and in energy metabolism [1]. β-Receptor blockers and inhibitors of the renin–angiotensin system are commonly used to prevent progression of left ventricular remodeling and hence development of heart failure due to condition such as myocardial infarction (MI). Nevertheless, a certain number of patients is still suffering from heart failure. Therefore, a better comprehension of the mechanism underlying pathological left ventricular remodeling is invaluable for the development of new therapeutic strategies.

In the setting of adverse left ventricular remodeling, the alteration of ECM components and the degradation by matrix metalloproteinases (MMPs) lead to progressive

Journal of Hypertension 2019, 37:1861–1870

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Received 13 October 2018 **Revised** 10 February 2019 **Accepted** 3 March 2019

J Hypertens 37:1861–1870

DOI:10.1097/HJH.0000000000002097

collagen deposition [2]. Recently, our group for the first time demonstrated the pathophysiological importance of TN-C, a large glycoprotein of ECM, in the progression of fibrosis and left ventricular hypertrophy in mice subjected to chronic pressure overload [3]. Other investigators have reported the pivotal role of TN-C in progression of myocardial and vascular remodeling [4,5] In addition, the elevated levels of TN-C in serum and myocardial tissue samples are accompanied by adverse left ventricular remodeling following MI [6,7]. Taken together, the enhanced levels of TN-C in infarcted and hypertrophied myocardium have been accompanied by left ventricular dilation and worse clinical outcome. Interstitial fibroblasts in the vicinity of the injured CMCs are the major source of TN-C and cumulative data demonstrated that inflammatory cytokines such as TGF-β, IL-1β, hypoxia and mechanical stress, exacerbate the synthesis of TN-C [8]. In addition, the loosening of cell adhesion and upregulation of MMPs following MI contribute to slippage of myocytes within the left ventricular wall, resulting in left ventricular wall thinning and dilatation, a mechanism at least partially driven by TN-C. Moreover, TN-C is also synthesized by endothelial and vascular smooth muscle cells as well as more recently shown by CMC [9]. Accordingly, Ma *et al.* [10] demonstrated that low but comparable levels of expression of long transcripts of TN-C between neonatal CMC and fibroblast under basal conditions was presented. Consistent with these findings, CMC and fibroblasts co-cultures treated with angiotensin II (Ang II) markedly enhanced TN-C expression, indicating the potential regulatory role of Ang II on TN-C expression. However, the mechanisms underlying the interactions between Ang II and TN-C in CMC remain unclear. More recently, adverse left ventricular remodeling is associated with epigenetic modifications, including microRNAs and DNA methylation, which play a pivotal role in left ventricular hypertrophy and fibrosis [11,12]. Accordingly, overexpression of mir-335 markedly reduced the expression of TN-C in hepatic stellate cells [13] and miR-335 suppressed metastasis through targeting of TN-C in breast cancer cells [14]. Nevertheless, the expression profiles of mir-335 in myocardial tissue samples after MI are unexplored. Therefore, we aimed to investigate the effects of hypoxic and hypertrophic stimuli on TN-C expression in H9c2 cells and in rats subjected to MI, and its putative regulation by epigenetic mechanisms, namely DNA promoter methylation and microRNAs.

MATERIALS AND METHODS

Rat model of myocardial infarction *in vivo*

Male Sprague–Dawley rats (10–12 weeks old, Department for Laboratory Animal Science and Genetics, Himberg, Austria) were anaesthetized by intraperitoneal injection of a mixture of Xylazin (4 mg/kg; Bayer, Leverkusen, Germany) and Ketamin (100 mg/kg; Dr E. Gräub AG, Bern, Switzerland), intubated and ventilated. Rats were subjected to permanent ligation of left coronary artery (LCA, MI group; $n = 8$) or Sham ($n = 4$) as described previously [15]. The experimental protocol was approved by the Ethics Committee for Laboratory Animal Experiments at the Medical University of Vienna and the Austrian Ministry of Science, Research and Economy (BMWFW-66.009/0023-WF/V/3b/2016) and in conformation with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Transthoracic echocardiography

Transthoracic echocardiography was performed on the anesthetized animal (isoflurane 2–3%) using a Vivid7 system GE Healthcare echocardiography machine equipped with an 11.5 MHz 10S sector transducer. Briefly, rats were placed in a supine position and an echocardiography probe was placed in gentle contact with the chest. Parasternal short axis views of the left ventricular cavity were collected at the level of the papillary muscle. M-mode echocardiography was performed. Based on M-mode images, left ventricular ejection fraction (%), as a primary end-point was assessed in three consecutive beats and averaged. Echocardiography was performed prior to and 7 days following MI in each experimental group.

Histology

Myocardial histology was performed on samples taken 7 days after MI induced in each group. Briefly, hearts were explanted washed and perfused with saline before being transferred to a 4% paraformaldehyde solution for 24 h. After this fixation period, heart sections (5 μm) were stained with hematoxylin and eosin and mounted on glass slides. In addition, heart sections were stained with Masson–Goldner to measure collagen contents. Images were acquired by microscopy (Olympus VS120 Virtual Slide Microscope System; Olympus, Tokyo, Japan) and captured by digital camera (AVT PIKE F-505C VC 50; Allied Vision Technologies, Stadtroda, Germany).

In-vitro H9c2 cardiomyoblast culture

H9c2 cardiomyoblasts (ATCC CRL-1446, Manassas, Virginia, USA) were cultured using M199 complete media [M199 media supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, and 1% L-glutamine (Thermo Fisher Scientific, Waltham, Massachusetts, USA)] as previously described [3].

Chronic hypoxia as a model of ischemic injury on H9c2 cells

H9c2 cardiomyoblasts were submitted to periods of oxygen glucose deprivation (OGD). Hence, 24-well imaging fluorocarbon (FC, MoBiTec GmbH, Goettingen, Germany) plates were coated with 2% gelatin for 1 h and 1×10^6 cells/ml were seeded with M199 complete media. Plates were then cultured under OGD or normoxic conditions at 37°C for 6 and 16 h. Afterwards, cardiomyoblasts were isolated for assessing cell viability or analysis of RNA as well as protein content.

Cardiomyoblasts in-vitro hypertrophy model

H9c2 cardiomyoblasts were incubated with hypertrophic inducers as described previously [16]. Briefly, M199 complete media were supplement with 1 μmol/l of Ang II (Sigma Aldrich, St Louis, Missouri, USA) for 48 h. Controls

were identically treated with M199 complete media, and total RNA was isolated.

The effect of exogenous human tenascin C on cellular hypertrophy

H9c2 cardiomyoblasts were exposed to two different concentrations of human TN-C purified protein (hTN-C; Merck & Co., Burlington, Massachusetts, USA). M199 complete media supplemented with 1 or 10 μg/ml of hTN-C was added for 24 and 48 h. Controls were also generated using M199 complete media. Total RNA was isolated and gene expression analyzed.

Total RNA and miRNA isolation

Total RNA and miRNA were isolated from H9c2 rat cardiomyoblasts, as well as from rat myocardium tissue samples (infarcted area and border zone, depicted in Fig. 1b). RNeasy Mini kit (Qiagen, Hilden, Germany) and miRNeasy kit (Qiagen) were used to isolate mRNA and miRNA respectively according to instructions.

Reverse transcription and quantitative polymerase chain reaction

cDNA was prepared using QuantiTect reverse transcription kit (Qiagen). Samples were analyzed in duplicate in a volume of 20 μl/well. The initial denaturation step of 15 min at 95°C was followed by 45 cycles of 15 s 95°C, 30 s 50°C and 30 s 72°C, using Rotor-Gene Q (Qiagen) and Rotor-Gene Q series software for Ct value analysis. Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method

Analysis of DNA methylation by methylation-specific polymerase chain reaction

Genomic DNA (gDNA) from H9c2 cells was obtained using the DNeasy Blood and Tissue Kit according to the manufacturer's instructions (Qiagen). One microgram of gDNA was denaturated and unmethylated cytosines converted to uracil in the denatured samples (Cells-to-CpG Bisulfite Conversion Kit; Thermofisher Scientific). CpG methylation was quantified by methylation-specific real-time PCR using 100 ng of bisulfite-converted genomic DNA as the template

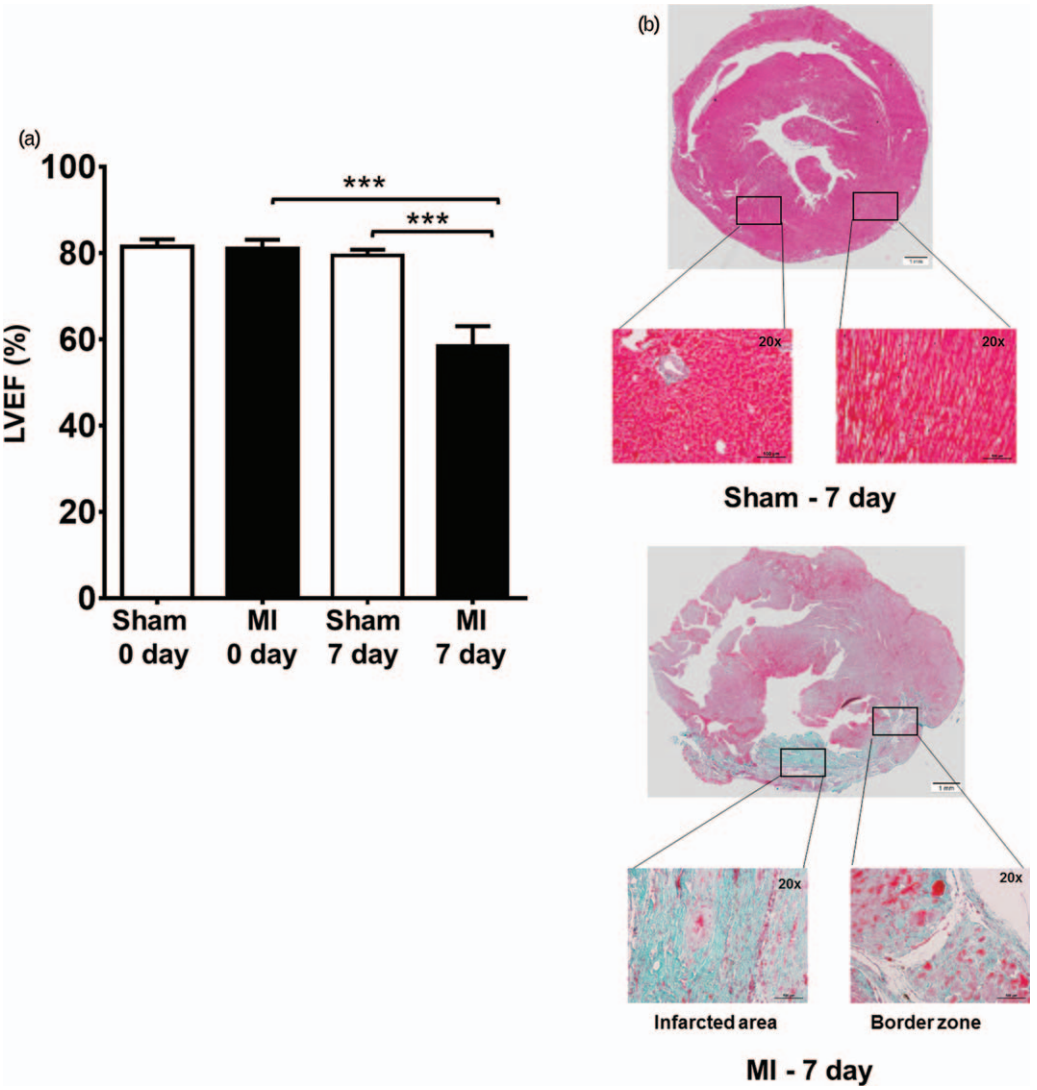

FIGURE 1 (a) Left ventricular ejection fraction (LVEF) prior to myocardial infarction (MI) and 7 days post MI. (b) Representative Masson-Goldner staining on left ventricular slices were obtained on day 7 after MI with showing infarcted and border zone. Data are presented as mean ± SD; $n = 4–7$. MI. $n = 4–7$; $^{***}P < 0.001$.

and methylation-specific primers for CpG in the human TN-C promoter. The methylation level of TN-C promoter was calculated by using the methylation index, as previously reported [17].

Reverse transcription and quantitative polymerase chain reaction for miRNAs

cDNA synthesis was prepared using TaqMan miRNA reverse Transcription kit according to instructions (cat. no. 4366596 from Thermo Fisher Scientific). qPCR was performed using TaqMan universal Master mix UNG (cat. no. 4324018 from Thermo Fisher Scientific), and mir-335 primer (has-miR-335, assay ID 4427975 from Thermo Fisher Scientific). Normalization was performed to Ubiquitin 6 (U6 snRNA, assay ID 001973 from Thermo Fisher Scientific). The thermocycler program was set to 10 min at 95°C and 40 cycles of 95°C for 10 s and 60°C for 55 s using Rotot-Gene Q (Qiagen). Rotor-Gene Q series software was used for Ct value analysis and relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method.

Viability assessment by annexin V/propidium iodide staining and flow cytometry analysis

The H9c2 cells were stained with fluorescein isothiocyanate-annexin V and propidium iodide (Becton, Dickinson Austria GmbH, Schwechat, Austria) according to manufacture, in order to determine the relative quantity of viable, early apoptotic, late apoptotic and necrotic cells. Stained cells were analyzed on a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, California, USA).

Tenascin C protein levels assessment by ELISA

Rat Tenascin C ELISA kit from CUSABIO (CSB-E13377r; Houston, Texas, USA)) was used according to the manufacturer instruction. Cellular protein concentration of TN-C following 6 and 16 h of OGD and plasma TN-C levels at 7 days post MI was quantified.

Measurement of high-energy phosphates

The sample preparation and high-performance liquid chromatography measurement of adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP) and phosphocreatine (PCr) were performed as previously described [18]. In brief, Untreated (control) or treated (Ang II; hTN-C) H9c2 cardiomyoblasts were used for the determination. After trypsinization and mild centrifugation (supernatant discarded) cellular proteins were precipitated with 250 μl of perchloric acid (0.4 mol/l). After centrifugation (12 000×*g*), 150 μl of the supernatant was neutralized with 15–20 μl of potassium carbonate (2 mol/l, 4°C). The supernatant (20 μl injection volume), obtained after centrifugation, was used for high-performance liquid chromatography analysis. Separation was performed on a Hypersil octadecylsilane column (5 μm; 250 × 4 mm I.D.) using an L-2200 autosampler, two L-2130 HTA pumps and an L-2450 diode array detector (all: VWR Hitachi, VWR, Vienna, Austria). Detector signals (absorbance at 214 and 254 nm) were recorded, and the software EZchrom Elite (VWR) was used for data acquisition and analysis. Energy charge was calculated according to the following formula: energy charge = (ATP + $^1/_2$ ADP)/(ATP + ADP + AMP). The pellets of the acid extract were dissolved in 1 ml of 0.1 mol/l sodium hydroxide and further diluted 1:10 with physiologic saline for protein determination [BCA (bicinchoninic acid) Protein Assay, Pierce, Thermo Fischer Scientific, Inc., Rockford, Illinois, USA].

Statistical analysis

Data are presented as mean ± SD. One-way ANOVA with Bonferroni post-hoc test was used for multiple comparisons between the groups and unpaired *t* test was used for comparison of two groups. $P < 0.05$ was considered statistically significant. Analysis was performed using Prism 6 software (GraphPad Inc., San Diego, California, USA).

RESULTS

Myocardial infarction resulted in upregulation of tenascin C

There was no difference in baseline left ventricular ejection fraction between the groups. However, MI ($n = 7$) resulted in a marked reduction in EF in comparison to baseline values and Sham-operated group ($n = 4$, $P < 0.001$) at 7 days post-infarction (Fig. 1a). In addition, Figure 1b depicted the extent of MI and shows the infarcted area and border zone. MI was accompanied by a significant increment of plasma TN-C concentration (Fig. 2a, $P < 0.01$). Moreover, TN-C mRNA expression was increased in the border zone when compared to the infarcted area (Fig. 2b). In parallel, there was a significant upregulation of cytokines such as TGF-β, IL-1β, whereas IL-6 expression remained unchanged (Fig. 2b). Interestingly, mir-335 expression was upregulated in the border-zone as compared to non-infarcted myocardial samples from Sham-operated rats (Fig. 2c, $P < 0.05$).

Hypoxia induces tenascin C formation in H9c2 cardiomyoblasts

Figure 3a shows a marked elevation of Tn-c expression following 6 h of OGD in comparison to the normoxic condition in H9c2 cells ($P < 0.05$). In addition, TN-C protein levels were measured following 6 and 16 h of OGD (Fig. 3b). Protein expression of TN-C was not detectable under normoxic conditions (data not shown). However, cells exposed to OGD showed an increase in TN-C protein concentration at both time points (Fig. 3b). Moreover, prolonged OGD did not significantly influence mir-335 expression in cells (Fig. 2c, $n = 4$).

Given the key role of DNA methylation in regulating gene transcription, we investigated TN-C promoter methylation after exposure to OGD in H9c2 cells. We found that hypoxia did not affect DNA methylation of the TN-C promoter as compared to control (Fig. 3d).

Prolonged OGD ultimately triggered cell death (Supplemental Figure 1, http://links.lww.com/HJH/B88, 50% decrease in viability after 16 h hypoxia). Nevertheless, H9c2 cells incubated with the hTN-C purified protein for either 24 or 48 h did not modify cells viability (apoptosis and necrosis; Supplemental Figure 2, http://links.lww.com/HJH/B88).

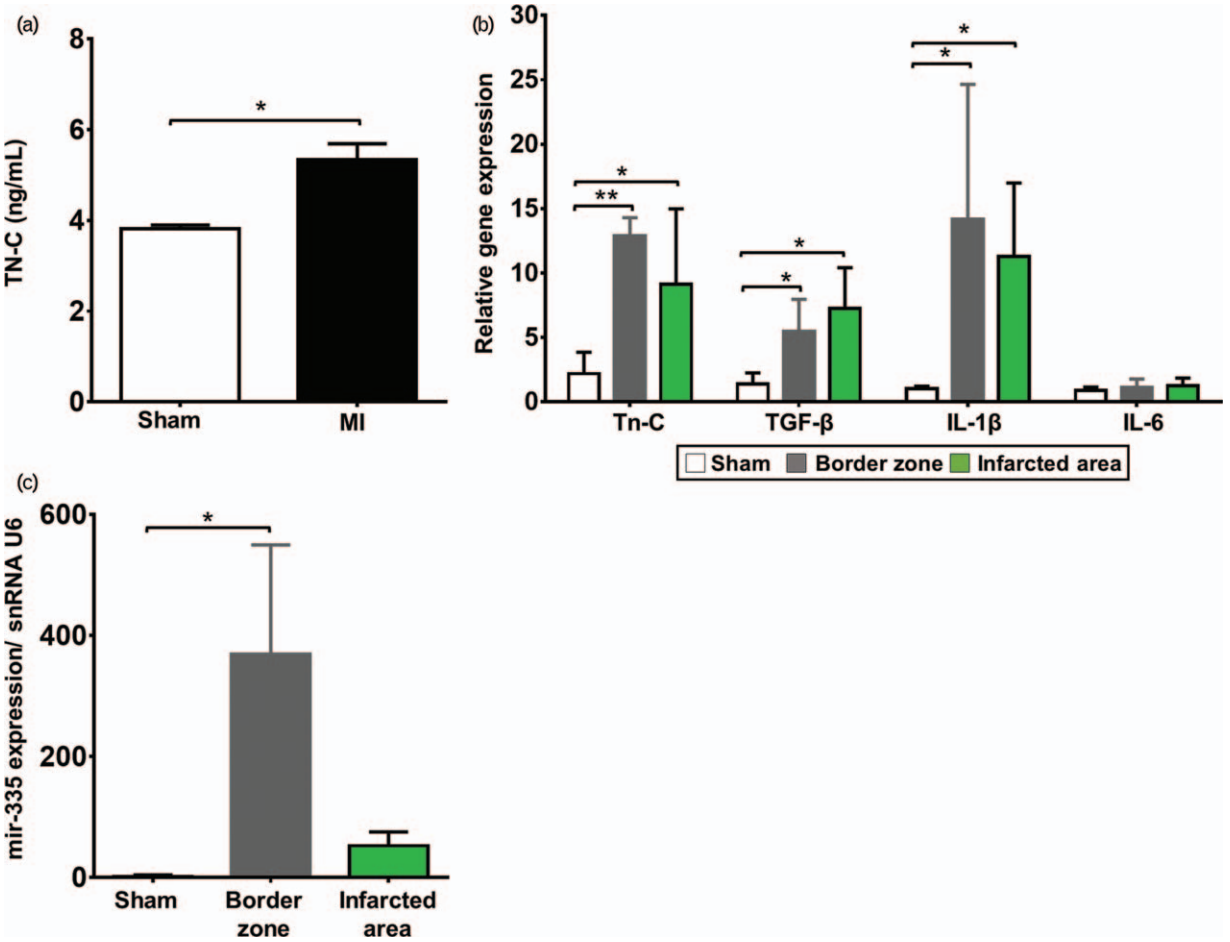

FIGURE 2 (a) Plasma tenascin C (TN-C) levels at 7-day post myocardial infarction (MI) measured by ELISA in Sham-operated (Sham) and MI group. (b) Relative mRNA expression of, Tn-c, Tgf-β, IL1-β and IL-6 and (c) mir-335 expression in left ventricular tissue samples obtained from the border zone (grey bar) and the infarcted area (green bar). Data are presented as mean ± SD; $n = 4$–7. MI. $n = 4$–7; $^{*}P < 0.05$, $^{**}P < 0.01$.

Epigenetic regulation of tenascin C by angiotensin II

H9c2 cells incubated with Ang II (1 mol/l) showed an upregulation of Bnp and Tn-C mRNA expression (Fig. 4a, $P < 0.05$ and $P < 0.01$, respectively) as well as a slightly increase of β-Mhc expression (Fig. 4a). In addition, H9c2 cells incubated with Ang II also showed an upregulation of mir-355 (Fig. 4b, $P < 0.05$). It is important to note that cells exposed to Ang II exhibited a reduced demethylation of TN-C promoter (Fig. 4c, $P < 0.01$).

Tenascin C regulates genes involved in left ventricle remodeling

TN-C is a highly conserved protein, maintaining its homology among species. H9c2 cells were incubated with hTN-C purified protein for either 24 or 48 h. H9c2 cells incubated with hTN-C markedly increased the expression of Bnp and β-Mhc after 48 h (Fig. 5a, $P < 0.001$) as well as time and dose dependently modified integrin α6 after 48 h incubation (Fig. 5b, $P < 0.01$). Integrin β1 expression was slightly increased after 48 h incubation with hTN-C (Fig. 5b). Mmp-2 expression was markedly increased in cells after incubation with hTN-C (Fig. 5a). hTN-C administration also initiated a temporary upregulation of TgfF-β (Fig. 5c, $P < 0.01$). In line with this, dysregulation of Smad 3 and 4 was observed after incubation of the cells with hTN-C (Fig. 5c, $P < 0.05$).

Tenascin C impairs cellular energy metabolism in H9c2 cells

Cells incubated with hTN-C showed a marked reduction in high-energy phosphate concentration, namely ATP (Fig. 6a, $P < 0.05$) and AMP (Fig. 6a, $P < 0.05$). Moreover, both Ang II and hTN-C-treated cells showed a tendency towards the decrease of phosphocreatine (PCr) (Fig. 6a). Energy charge was similar between all groups (Fig. 6b).

DISCUSSION

We investigated the effect of hypoxia and hypertrophic stimuli on TN-C expression in H9c2 cells and its putative regulation by epigenetic mechanisms, namely DNA promoter methylation and microRNAs. Both conditions lead to an upregulation of TN-C in H9c2 cells. In addition, TN-C directly regulated genes involved in progression of left ventricular hypertrophy and heart failure, such as Mmps, Bnp and integrins. Furthermore, Ang II led to reduced demethylation of the TN-C promoter, subsequently

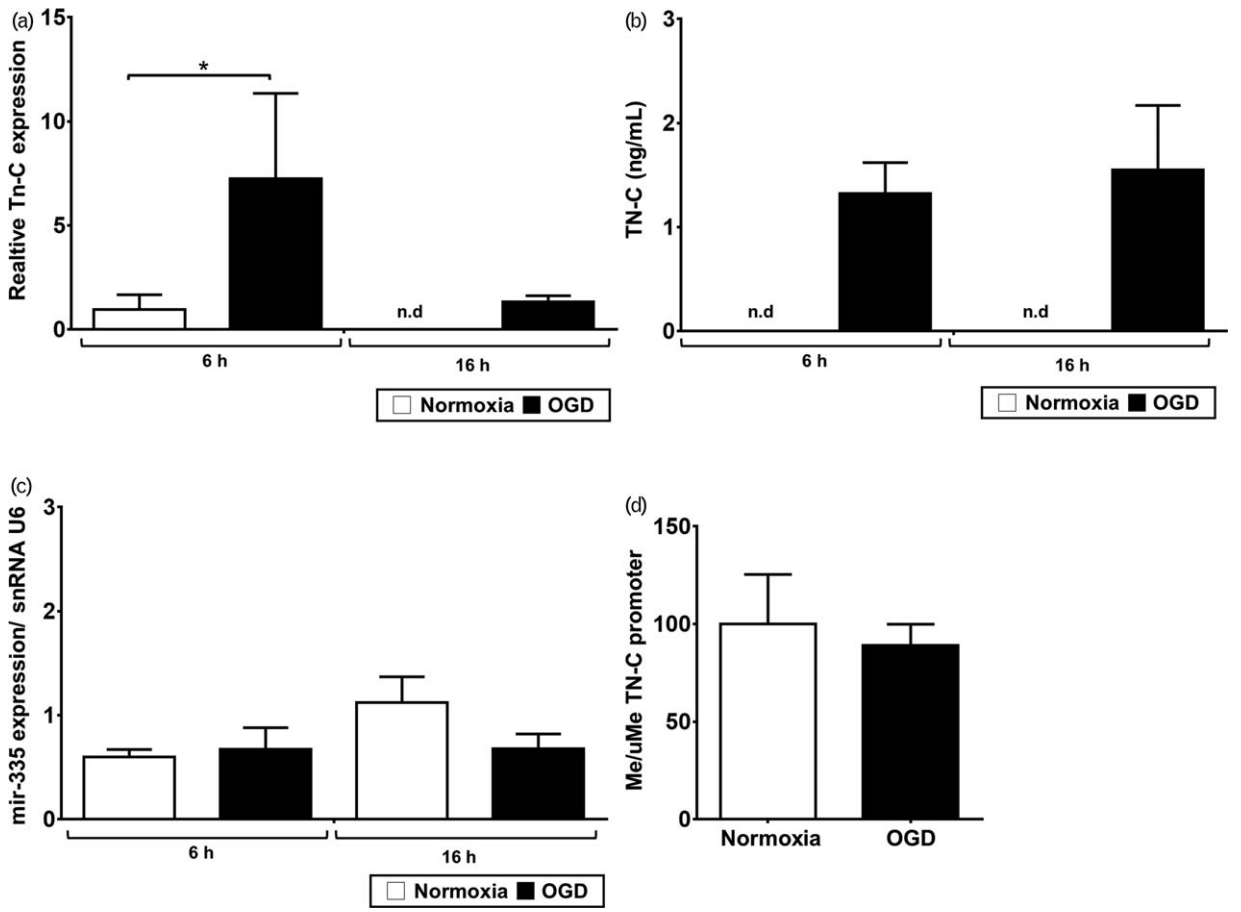

FIGURE 3 (a) Tenascin C mRNA and (b) tenascin C (TN-C) protein expression following 6 and 16 h of oxygen and glucose deprivation (OGD). (c) mir-335 expression in H9c2 cells exposed to OGD. (d) TN-C promoter methylation following OGD. n.d., not detected. Data are presented as mean ± SD; $n = 4$–6.*$P < 0.05$.

resulting in TN-C upregulation in CMCs. This TN-C upregulation was accompanied by an enhancement in plasma and tissue of TN-C levels in rats subjected to chronic MI.

Adverse left ventricular remodeling due to MI is accompanied by substantial growth on myocyte size, dysregulation of cytoskeleton genes, overactivation of renin angiotensin aldosterone system, ECM components and re-expression of fetal genes [19]. Notably, re-expression and transient upregulation of TN-C in post-MI culminates in worse clinical outcome [6,7,20], suggesting the pathophysiological importance of TN-C. Accordingly, recent studies demonstrated that the peak of TN-C upregulation in left ventricular tissue samples (border zone and infarcted area) occurred 5–7 days post-MI [7,21]. In line with these studies, we found that both circulating and tissue levels of TN-C was markedly increased at 7 days post-MI. In addition, H9c2 cells exposed to OGD showed TN-C upregulation. These results are also supported by a recent study [10] which demonstrated that besides fibroblasts, CMCs are also capable of producing TN-C under certain conditions. Therefore, in our study we focused on the impact of TN-C on CMCs rather than fibroblast. Accordingly, H9c2 cells exposed to hTN-C showed an upregulation of both hypertrophic and fibrotic markers such as Bnp and Smad3.

There is substantial evidence that Tgf-β signaling is an essential player in inflammatory response and healing after MI. We found an enhancement of Tgf-β expression in infarcted myocardium from rats subjected to chronic ligation of LCA. In line with this findings, Tn-C expression was also upregulated 7 days post-MI, which may indicate a potential interaction of Tn-C and Tgf-β [22]. Accordingly, H9c2 cells exposed to hTN-C for 24 h showed a marked upregulation of Tgf-β. Tgf-β signaling activation by TN-C in CMC might provide a pro-survival signal and subsequently lead an improvement of infarct healing [23]. Upregulation of Tgf-β expression contributes to CMC apoptosis [23] and promotes fibroblast proliferation as well as ECM degradation. Therefore, this upregulation of TGF-β plays a central role in maladaptive post-MI remodeling.

We also investigated the possible epigenetic modifications – including, non-coding RNAs (mir-335) and DNA methylation on TN-C expression. Recently, it has been demonstrated that microRNAs play a significant role in left ventricular remodeling, in terms of increasing interstitial fibrosis and activating ECM degradation [24]. However, only a few studies have investigated the epigenetic control of TN-C. Chen *et al.* [25] showed that mir-335 is involved in the regulation of TN-C expression in hepatic satellite cells. In addition, mir-335 suppresses the ECM component TN-C which directly targets the 3′ UTR of TN-C [14]. In our OGD cellular model, the expression level of mir-335 remained unchanged, whereas Tn-C was upregulated. In contrast, myocardial tissue samples from either border zone or infarcted area showed a massive upregulation of mir-335.

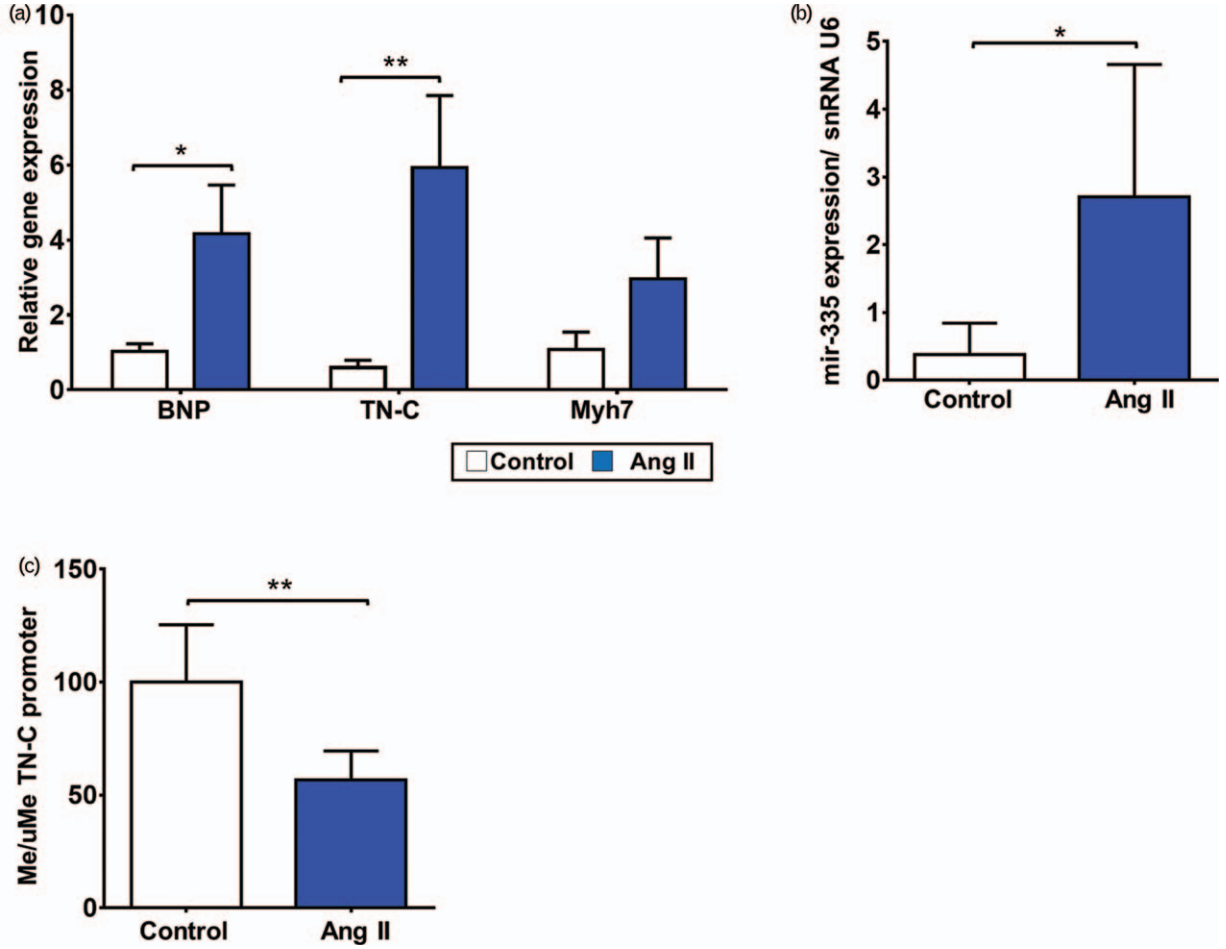

FIGURE 4 (a) Bnp, tenascin C, and β-Mhc expression in H9c2 cells following 48 h incubation with 1 μmol/l of angiotensin II (Ang II). (b) Effect of Ang II on mir-335 expression in H9c2 cells. (c) Tenascin C (TN-C) promoter methylation after incubation with Ang II for 48 h. Data are presented as mean ± SD; $n = 4$–6. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Simultaneously, the mRNA expression of Tn-C within the same areas was also increased. Based on the inconsistent data between our results and previous findings, we interpret the discrepancies as follows. We used a different cell type which implies that there is possible functional difference in regulation of Tn-c by mir-355 in comparison to tumor cells and cardiomycyte (H9c2 cells). This is supported by the findings that we did not observe an up regulation or downregulation of mir-335 expression in H9c2 cells after OGD exposure. However, cells exposed to OGD shown an increase in Tn-C expression. Recently, Zhu *et al.* [26] have demonstrated that mir-335 promotes upregulation of pro-inflammatory cytokines such as TNF-α and IL-6 in human mature adipocytes. These cytokines also play a significant role in adverse left ventricular remodeling post-MI.

In fact, activation of the adrenergic system with associated upregulation of the renin–angiotensin–aldosterone system, including Ang II play a major role in progression of adverse left ventricular remodeling [27,28]. Notably, we found that H9c2 cells exposed to Ang II resulted in reduced DNA methylation of the TN-C promoter, indicating that Ang II is a potential regulator of TN-C. Moreover, promoter methylation is an important repressor of gene transcription, while reduced methylation of CpG sites favors chromatin accessibility to transcription factors [29]. We observed changes in TN-C promoter methylation with Ang II but not with OGD, suggesting that different stimuli (OGD vs. Ang II) may activate different epigenetic networks. The growing importance of methylating agents in clinical practice [30] highlight the possibility to restore TN-C expression by targeting enzymes involved in DNA methylation, namely DNMT3a and 3b.

Adverse left ventricular remodeling is associated with a marked left ventricular dilatation. In our previous study TN-C-KO mice showed preserved left ventricular function which was accompanied by a significantly reduced left ventricular dilatation [3]. Dysregulation of integrin β1 has been demonstrated to play a role in CMC apoptosis [31] and left ventricular dilatation [32]. Furthermore, Hessel *et al.* [33] found that pressure overload-induced right ventricular failure was associated with TN-C upregulation and concomitant reduction of integrin α6. Accordingly, we investigated the impact of TN-C on integrin β1 expression in H9c2 cells. Cells exposed to hTN-C for 24 h showed decreased mRNA expression of integrin β1 when compared to untreated controls. Interestingly, we found that integrin α6 was markedly increased after 48 h incubation with hTN-C. This rise in the integrin α6 levels may also contribute to left ventricular dysfunction. In addition, TN-C increased the

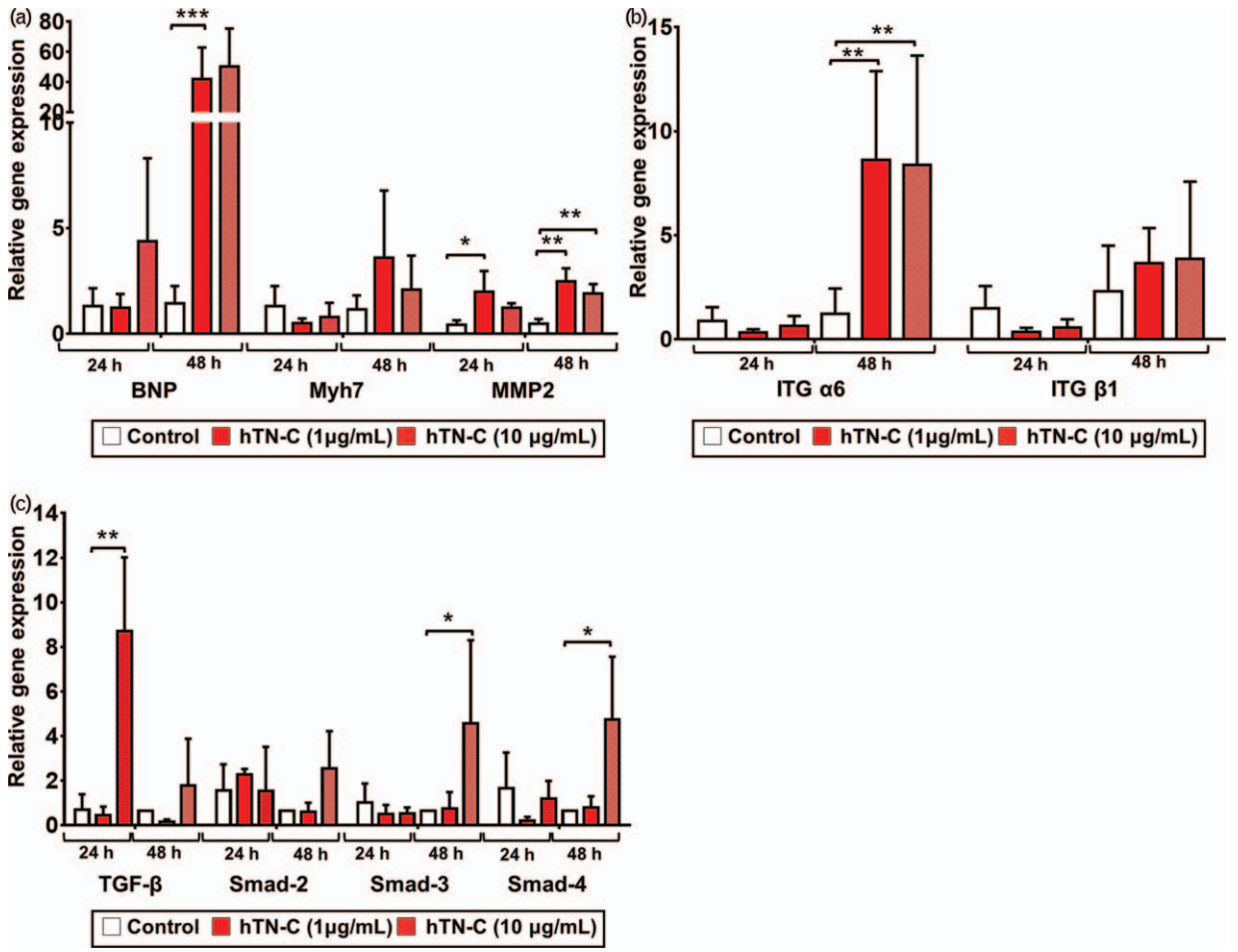

FIGURE 5 (a) BnpP, β-Mhc and Mmp-2 expression after 24 or 48 h incubation with 1 and 10 µg/ml of human tenascin C (TN-C) (hTN-C). (b) mRNA expression of Itg α6 and Itg β1 after 24 and 48 h incubation with 1 and 10 µg/ml of hTN-C. (c) Effect of hTN-C on Tgf-β and Smad 2, 3 and 4 mRNA expression. Data are presented as mean ± SD; $n = 4$–6. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

expression of Mmp-2 in H9c2 cells, which further underline the pathophysiological importance of TN-C in adverse left ventricular remodeling and left ventricular dilatation. Besides integrin, Ang II accelerates left ventricular dilation acting on Ang II receptor [34]. Accordingly, we found an Ang II-dependent TN-C upregulation on epigenetic levels.

Finally, we investigated the impact of TN-C on cellular energy metabolism. Of note, dysregulation of myocardial metabolic processes appears to be a major contributor to heart failure pathogenesis [35]. By addition of hTN-C to H9c2 cells, a significant decrease in ATP formation was observed, revealing an impaired mitochondrial activity as

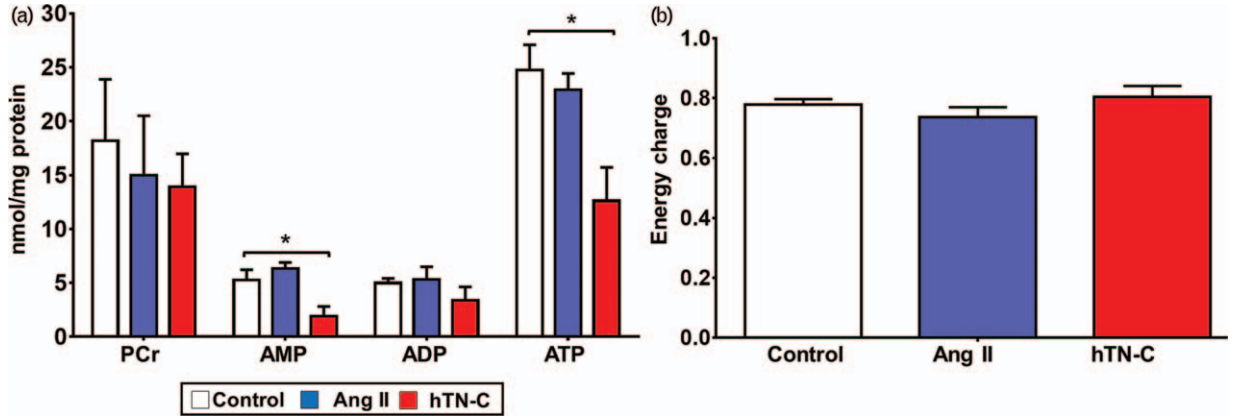

FIGURE 6 (a) Effect of human tenascin C (TN-C) (hTN-C) and Ang II treatment on high energy phosphates levels and (b) energy charge after 48 h incubation (hTN-C: 10 µg/ml; Ang II: 1 µmol/l) in H9c2 cells. Data are presented as mean ± SD; $n = 6$. $^{*}P < 0.05$.

well as in consequence a reduction of cardiac function. Similarly, TN-C significantly reduced the levels of AMP as well as in the total pool of adenine nucleotides. A key player involved in regulating myocardial metabolism is AMP-activated protein kinase (AMPK), a regulatory kinase controlling numerous metabolic pathways and deficiency of AMPK boosts left ventricular dilatation [36]. Nevertheless, further studies are warranted to explore the interaction of TN-C and AMPK in dilative cardiomyopathy.

Several limitations of the present study have to be considered. First, studies of aberrant molecular signaling process occurring during left ventricular hypertrophy mostly use CMCs from neonatal rodent hearts. In the current study, we used the H9c2 cardiomyoblast cell line. However, previous studies have demonstrated that H9c2 cells and rat neonatal CMCs show almost an identical hypertrophic response to Ang II [16]. Second, we only focused on mir-335 and studied the regulatory role of this miRNA on TN-C regulation. Nevertheless, our study to the best of our knowledge for the first time showed that mir-335 is upregulated in post MI myocardium. Third, we studied the regulatory role of Ang II on TN-C promoter in CMCs. However, major source of TN-C during myocardial remodeling are fibroblast. Therefore, further studies are warranted to clarify the regulatory role of Ang II on promoter activity in fibroblast or other cells.

In conclusion, our study underlines the potential pathophysiological importance of TN-C on the CMCs level. Tn-C was found to be upregulated in H9c2 cells under the influence of OGD or Ang II. This was accompanied by a dysregulation in the expression of ECM components such as Mmp-2 and the cell adhesion receptors integrins. In addition, Ang II led to a reduced demethylation of TN-C promoter and H9c2 cells exposed to hTN-C showed an impairment in energy metabolism. These findings may contribute to our understanding of TN-C regulation in the heart, thus encouraging new approaches to prevent adverse left ventricular remodelling in patients with MI.

ACKNOWLEDGEMENTS

Professor Andreas Spittler from Anna Spiegel Core Facilities, Medical University of Vienna, Austria, for establishing FACS protocol. The authors would like to thank Gerd Kager for the support by the measurement of HEP (Medical University of Graz, Austria) and Milat Inci for technical assistance (Medical University of Vienna, Austria). Francesco Paneni is the recipient of a Sheikh Khalifa's Foundation Assistant Professorship at the Faculty of Medicine, University of Zürich.

This study was supported by the Ludwig Boltzmann Cluster for Cardiovascular Research, Vienna, Austria (REM, 2017-2020 project).

Conflicts of interest

There are no conflicts of interest.

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